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(19)

(54) VIRAL ASSAY

(71) We, MERCK & CO. INC., a corporation duly organized and existing under the laws of the State of New Jersey, United States of America, of Rahway, New Jersey, United States of America, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention is concerned with assays for viral infectivity and for determining the content of serum-neutralizing antibodies.

The present invention provides a method of determining the presence or absence of the cytopathic effect, without optical magnification, in a cell culture that has been infected with a virus, comprising incubating the infected cell culture for a time sufficient to permit a development of cytopathic effect, staining the cell culture with a protein stain effective to stain and fix the cells substantially immediately, exposing the stained cell culture to a light source and determining whether the cell culture contains a clear area through which light passes. The protein stain used is one like carbolfuchsin that is effective to stain proteins and that can simultaneously stain and kill the cells.

In a practical method of carrying out the method, dilutions of a virus preparation, preferably prepared electro-mechanically, are mixed with a suspension of the appropriate tissue culture-grown cell suspension which, preferably, has been automatically pipetted into the wells of a microtiter plate. Following the said incubation of the cells in the plate under appropriate conditions, fluids are drained from the plate and the cells are stained with a protein stain and then read without optical magnification by exposure to the light source.

The present invention also provides a method of determining antibody content of serum comprising the steps of

(a) automatic dilution of the serum samples;
(b) automatic addition of a challenge virus to the samples;
(c) preincubation of the serum/virus mixtures;
(d) automatic delivery of the serum/virus mixtures to a cell suspension;
(e) concurrent infection and formation of cell sheet during incubation of the cells in the suspension;
(f) draining fluids from the culture; and
(g) staining with a protein stain effective to stain and fix the cells substantially immediately and determination of the cytopathic effect without optical magnification.

In a practical method of carrying out this assay, dilutions of a serum sample being assayed are mixed, preferably electro-mechanically, with the challenge virus. To an appropriate tissue-culture-grown cell suspension, which preferably has been automatically pipetted into the wells of a microtiter plate, is added the preincubated serum/virus mixture. Following incubation of the cells in the microtiter plate under appropriat conditions, fluids are drained from the plates and the cells are stained with a protein stain and then read without optical magnification.

The viral assay of the present invention is applicable to any virus. It can be used for viruses which affect humans as well as for viruses which affect animals. Some examples of viruses that can be used in the viral assay of the present invention are rubella, measles, mumps, herpes, polio, varicella and Marek's.

I. VIRAL ASSAY

A. Preparation of Assay Plates

Sterile tissue culture plates, transfer plates and lids are removed from their wrappers and assembled with the transfer plate in place in the tissue culture plate, both covered by the lid. The plates are marked with the cells being used, the date the assay is being started and an individual identification number on both lid and tissue culture plate. An assay sheet is prepared for each sample.

B. Preparation of Samples

Frozen samples and an aliquot portion of the house reference standard are thawed rapidly by being placed in a bath partially filled with cold tap water. The samples are thawed just prior to use and are kept in the cold water bath until removed for assay.

Lyophilized samples are reconstituted by adding required amount of diluent and kept in the cold water bath until removed for assay.

C. Preparation of Pipetter

A disposable plastic reservoir is placed in position in the base of the automatic pipetter. The pipetting head is partially placed in position in the unit after which a rubber vacuum diaphragm is positioned on top of the pipetting head. The diaphragm-covered pipetting head is then moved into its final position and locked in place. 70 ml of diluting medium is then pipetted, using a sterile 100-ml volumetric pipette, into the reservoir, which is refilled as required.

D. Preparation of Diluter

A disposable plastic reservoir is filled with sterile distilled water to a depth of approximately $\frac{1}{4}$ inch (sufficient for total immersion of the tips of the microdiluters).

The assembly of microdiluters is removed from the diluter, immersed briefly in the water and touched down on a blotter, and then each microdiluter is burned off in the flame of a Bunsen burner. The heated microdiluters are then dipped again into the water, touched down on the blotter and repositioned in the diluter.

After every plate, the assembly of microdiluters is removed, touched down on the blotter to remove residual virus suspension, immersed in the water rinse and then blotted. (The cycle of immersion and blotting is reapeated two more times). After every third plate, the assembly of microdiluters is removed, touched down on the blotter, immersed in water, blotted, flamed as described above, immersed and blotted. This same procedure is used at the end of the day's assays.

E. Titration of Samples

The transfer plate is removed from the tissue culture plate, transfer plate and lid assembly and placed in the transfer plate holder. (The lid is replaced on top of the tissue culture plate.) The transfer-plate holder is positioned in the automatic pipetter and 0.075 ml of diluent (0.025-ml drops 3 times) is added to each of the wells, typically 96 wells. The transfer-plate holder is then removed from the pipetter. One drop (0.025 ml) of the sample being tested is carefully added, using a sterile pipette, to each of the 12 wells in Row A of the transfer plate. A fresh pipette is used for each sample. The transfer-plate holder is then positioned in the automatic diluter which is then operated as indicated in the manufacturer's instructions. This performs seven 1:4 serial dilutions (0.6 $\log_{10}$ per dilution). The transfer plate is then removed from the transfer-plate holder and placed back in the tissue culture plate and covered with the lid. If the sample is likely to have a titer greater than 4.2 $\log_{10}$, it should be diluted at least 10-fold below that level immediately before assay, using diluent.

When all the samples and reference standards have been diluted as above, the cell suspension is added as follows: the transfer plate is removed from the assembled unit and placed in the transfer-plate holder and covered with the lid. The tissue-culture plate of the unit is positioned in the automatic pipetter. (Just prior to this, the disposable reservoir containing diluent is replaced with a disposable reservoir containing the cell suspension at a concentration of from

about 160,000 to about 260,000 cells per ml, preferably about 200,000 cells per ml. The pipetter is filled and emptied into the reservoir a few times to flush the unit.) A quantity, 0.075 ml, of the cell suspension is added to each of the wells in the tissue-culture plate (0.025 ml drops 3 times).

The tissue-culture plate is removed from the pipetter and the transfer plate is placed in it and then lifted out, permitting of transfer of the virus dilutions in the transfer plate to the cell suspension in the tissue-culture plate. The transfer plate is discarded (to be autoclaved) and the lid is fitted carefully over the tissue-culture plate. The other plates are treated similarly and stacked above one another, then placed in the incubator at the requisite incubating temperature, typically from 30° to 39°C, for the optimal inbubation time for the virus.

At the end of the optimum incubation period for the particular virus, the plates are removed from the incubator. The contents are drained into a large pan by a sharp snap of the wrist, the wells pointing downward. Each plate is, in turn, positioned in the automatic pipetter, the reservoir of which has previously been filled with a protein stain, e.g., Carbolfuchsin stain.

The Carbolfuchsin stain may be used in concentrated form with a washing step following the staining, or in diluted form without a washing step following the staining. Then 0.075 ml of the stain is added to each well (0.025-ml drops 3 times). After half a minute or more the stain is drained from the plate (as described above). The plate is immersed in a deep basin of tap water to rinse out excess of stain and drained as before. (Rinsing may be eliminated by use of a more dilute stain). The plate is dried with a paper towel and covered with its lid.

F. Reading Assay

The plates are read macroscopically without optical magnification by exposure to a light source. A convenient method is to place the plates on a fluorescent light box. The wells showing CPE (cytopathic effect) are readily recognized by the areas showing no stain. Uninfected wells have a uniform red matrix. The infected wells are scored positive (+) and the uninfected wells negative (–) on the assay sheet.

To calculate the titer of the sample, the infected and uninfected wells are summed up for each line of the assay plate using a calculation sheet. As indicated on that sheet, the titer is determined by a Reed-Muench or Karber calculation—briefly, the negatives are added going down, the positives added going up and the percent positive calculated at each dilution level as shown in the sample calculation.

II. SERUM NEUTRALIZING ANTIBODY ASSAY

A. Preparation of Assay Plates

Sterile tissue culture plates, transfer plates and lids are removed from their wrappers and assembled with the transfer plate in place in the tissue culture plate, both covered by the lid. The plates are marked with the cells being used, the date the assay is being started and an individual identification number on both lid and tissue culture plate. An assay sheet is prepared for each sample.

B. Preparation of Samples

The sera to be assayed are inactivated for 30 minutes at 56°C and cooled to room temperature prior to use.

C. Preparation of Pipetter

A disposable plastic reservoir is placed in position in the base of the automatic pipetter. The pipetting head is partially placed in position in the unit after which a rubber vacuum diaphragm is positioned on top of the pipetting head. The diaphragm-covered pipetting head is then moved into its final position and locked in place. 70 ml of diluting medium is then pipetted, using a sterile 100-ml volumetric pipette, into the reservoir, which is refilled as required.

D. Preparation of Diluter

A disposabl plastic reservoir is filled with sterile distilled water to a depth of approximately ¼ inch (sufficient for total immersion of the tips of the microdiluters).

Th assembly of microdiluters is removed from the diluter, immersed briefly in the water and touched down on a blotter, and then each microdiluter is burned off in the flame of a Bunsen burner. The heated microdiluters are then dipped again into the water, touched down on the blotter and repositioned in the diluter.

After every plate, the assembly of microdiluters is removed, touched down on the blotter to remove residual virus suspension, immersed in the water rinse and then blotted. (The cycle of immersion and blotting is repeated two more times). After every third plate, the assembly of microdiluters is removed, touched down on the blotter, immersed in water, blotted, flamed as described above, immersed and blotted. This same procedure is used at the end of the day's assays.

E. Titration of Samples

The transfer plate is removed from the tissue culture plate, transfer plate and lid assembly and placed in the transfer-plate holder. (The lid is replaced on top of the tissue-culture plate.) The transfer-plate holder is positioned in the automatic pipetter and 0.025 ml of diluent (one drop) is added to each of the 96 wells. The transfer-plate holder is then removed from the pipetter. One drop (0.025 ml) of the sample (serum) being tested is carefully added, using a sterile pipette, to each of the two adjacent wells in Row A of the transfer plate and so on with five other sera being tested. The transfer-plate holder is then positioned in the automatic diluter, which is then operated as indicated in the manufacturer's instructions. This performs seven 1:2 serial dilutions. The transfer plate holder is then removed from the automatic diluter and positioned in an automatic pipetter, the reservoir of which is filled with the challenge-virus suspension. One drop (0.025 ml) of the challenge virus suspension is then added to each well of the transfer plate. The transfer plate containing the mixtures of the diluted serum samples and virus is then removed from the transfer-plate holder, placed on the tissue culture plate, covered with the lid and incubated for 1 hour at 36±1°C, 5% CO_2, 95% RH. At the end of the incubation period, the transfer plate is placed on the transfer plate holder and covered with the lid. The tissue culture plate is placed in the automatic pipetter the reservoir of which has been filled with an appropriate tissue culture suspension (typically 160,000 VERO cells per ml). 0.075 ml (3 drops) of the cell suspension is added for each of the wells in the tissue culture plate. The tissue-culture plate is removed from the pipetter and the transfer plate is placed in it and then lifted out permitting of transfer of the virus dilutions in the transfer plate to the cell suspension in the tissue-culture plate. The transfer plate is discarded (to be autoclaved) and the lid is fitted carefully over the tissue-culture plate. The other plates are treated similarly and stacked above one another, then placed in the incubator at the requisite incubating temperature, typically from 30° to 39°C, for the optimal incubation time for the virus.

At the end of the optimum incubation period for the particular virus, the plates are removed from the incubator. The contents are drained into a large pan by a sharp snap of the wrist, the wells pointing downward. Each plate is, in turn, positioned in the automatic pipetter, the reservoir of which has previously been filled with a protein stain, e.g., Carbolfuchsin stain.

The Carbolfuchsin stain may be used in concentrated form with a washing step following the staining, or in diluted form without a washing step following the staining. Then 0.075 ml of the stain is added to each well (0.025-ml drops 3 times). After a half-minute or more the stain is drained from the plate (as described above). The plate is immersed in a deep basin of tap water to rinse out excess of stain and drained as before. (Rinsing may be eliminated by use of a more dilute stain). The plate is dried with a paper towel and covered with its lid.

G. Serum Control

In order to ascertain whether the sera being tested are toxic to the cells used in the titration, another plate is prepared in which everything is the same except that diluent is substituted for the challenge virus.

H. Virus Titration

The challenge virus used in this procedure is simultaneously assayed as described in the Viral Assay Procedure. For the dilution used in the serum neutralization assay the virus contains 20 to 50 $TCID_{50}$ per 0.025 ml.

The following examples illustrate but do not limit the present invention. Unless indicated otherwise, all temperatures are expressed in degrees Celsius.

EXAMPLE 1.

A. Tissue Culture

Cell suspensions of a rabbit kidney continuous cell line are prepared on the day they are to be used in the assay at 200,000 cells per ml of EMEM (Eagle's Minimum Essential Medium) + 10% v/v fetal calf serum (not heat inactivated) + 1% v/v of a 200 milli-molar solution of *L*-glutamine + 0.05% of a 100 mg/ml solution of Neomycin (the *L*-glutamine is added just before use). Approximately 8 ml of cell suspension is required per sample. The cells are stirred until used.

B. Culture Medium

Diluting medium — EMEM + 2% v/v fetal calf serum (not heat inactivated) + 0.05% v/v of a 100 mg/ml solution of Neomycin + 1% v/v of a 200 milli-molar solution of *L*-glutamine (the *L*-glutamine is added just before use).

C. Carbolfuchsin stain, concentrated

10 ml fuchsin stock (10 g basic fuchsin stirred 15 minutes in 95 ml warm 95% ethanol),
70 ml ethanol, 95%,
320 ml phenol, 5% in water.

D. Virus Samples

1. Frozen samples are stored at −70° until tested.
2. Lyophilized samples are stored at 2—5° until tested.
3. Frozen aliquot portions of a house reference standard virus suspension are stored at −70° until tested. An aliquot portion is tested with each assay.

The assay plates, pipetter and diluter are prepared as indicated in the preceding Detailed Description. The samples of rubella virus, whether frozen or lyophilized, are prepared as indicated in section B of the Detailed Description. The sample is then added to the wells in row A of the transfer plate and the sample titrated, incubated and stained as in section E of the Detailed Description. The incubation is carried out at 32°±1°, 5% CO_2, 95% RH for 10 days. The staining is effected using concentrated Carbolfuchsin stain.

The plates are then read by being placed on the fluorescent light box and infected wells scored positive (+) and uninfected wells negative (–). The assay sheet is as follows:

	SAMPLE											
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	–	+	+	–	–	–	–	–	–	–	+	+
F	–	+	–	–	–	+	–	+	–	–	–	–
G	–	–	–	+	–	–	–	–	–	–	–	–
H	–	–	–	–	–	–	–	–	–	–	–	–

The titer of the sample is calculated using the Reed-Muench or Karber technique using the following calculation sheet:

	$\log_{10}$ dilution of sample	P/N	N	P	%P
A	0.6	12/0			
B	1.2	12/0			
C	1.8	12/0			
D	2.4	12/0	0	20	100
E	3.0	4/8	8	8	50
F	3.6	3/9	17	4	19
G	4.2	1/11	28	1	3
H	4.8	0/12	40	0	0

The titer of this sample is 3.0 $\log_{10}$ per 0.025 ml.

EXAMPLE 2.

Determination of Mumps-Serum-neutralizing Antibody

A. Tissue Culture

Vero (a cercopithecous monkey kidney continuous cell line) cell suspensions are prepared on the day they are to be used in the assay at 160,000 cells per ml in Medium 199 + 10% v/v fetal calf serum (not inactivated) + 0.05% v/v of a 100 mg/ml solution of Neomycin. Approximately 8 ml of cell suspension is required per plate. The cells are stirred until used.

B. Diluting Medium

Medium 199 + 20 ml per liter of agamma calf serum (inactivated) + 85 ml per litre of 2.8% $NaHCO_3$ + 0.05% v/v of a 100 mg/ml solution of Neomycin.

C. Carbolfuchsin stain, dilute

10 ml fuchsin stock,
175 ml ethanol, 95%,
800 ml phenol, 5% in water.

D. Serum samples

All samples are inactivated (56°, 30 minutes) prior to use.

E. Challenge virus

MSD Mumps House Standard No. 3 diluted 1:20 with diluting medium just prior to use (or other suitable mumps virus preparation).

F. Procedure

The assay plates, pipetter and diluter are prepared as indicated in the preceding Detailed Description. A transfer plate, held in the transfer plate holder, is positioned in th pipetter, and one drop (0.025 ml) of diluting medium is added to each of the 96 wells. One drop (0.025 ml) of the serum being tested is added to each of two adjacent wells in row A of the transfer plate and so on with the five other sera being tested. The transfer plate is then positioned in the automatic diluter which, when operated, performs seven serial 1:2 dilutions of the sera being tested. The transfer plate, in the holder is then repositioned in the pipetter (just

prior to this, the disposable reservoir has been filled with the suspension f the challenge virus) and one drop of challenge virus is added to each well. The transfer plate is then placed in the tissue culture plate, covered with the lid and kept in an incubator (36±1°, 5% CO_2, 95% RH) for 1 hour. At the end of that period, the transfer plate is removed from the assembled unit and placed in the transfer plate holder and covered with the lid. The tissue culture plate of the unit is positioned in the automatic pipetter (just prior to this, the disposable reservoir has been filled with the cell suspension) and 0.075 ml of the cell suspension is added to each of the 96 wells in the tissue culture plate. The tissue-culture plate is removed from the pipetter and the transfer plate is placed in the tissue-culture plate and then lifted out, permitting transfer of the mixtures of serum dilutions and virus in the transfer plate to the cell suspensions in the transfer plate. The transfer plate is discarded and the lid fitted over the tissue culture plate. Other plates are treated similarly and all plates are kept at 36±1°, 5% CO_2 for 7 days. The plates are stained using the dilute Carbolfuchsin stain, drained but not washed and read as described in Example 1.

(2) Serum control

In order to ascertain whether the sera are toxic to the cells used in the titration, another plate is prepared in which everything is the same except that diluting medium is substituted for the challenge virus.

(3) Virus titration

The challenge virus used in this procedure is simultaneously assayed, as described in Example 1. For the dilution used in the serum neutralization assay the virus contains 20 to 50 $TCID_{50}$ per 0.025 ml.

(4) Assay sheet

	Serum 1		Serum 2		Serum 3		Serum 4		Serum 5		Serum 6	
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	−	−	−	−	−	−	+	+	−	−
B	+	+	−	−	+	+	−	−	+	+	−	−
C	+	+	−	−	+	+	−	−	+	+	−	−
D	+	+	−	−	+	+	−	−	+	+	+	+
E	+	+	+	+	+	+	−	−	+	+	+	+
F	+	+	+	+	+	+	−	−	+	+	+	+
G	+	+	+	+	+	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	+	+	+	+	+

Calculati n of results

	Dilution	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6
A	1:2	2/0	0/2	0/2	0/2	2/0	0/2
B	1:4	2/0	0/2	2/0	0/2	2/0	0/2
C	1:8	2/0	0/2	2/0	0/2	2/0	0/2
D	1:16	2/0	0/2	2/0	0/2	2/0	2/0
E	1:32	2/0	2/0	2/0	0/2	2/0	2/0
F	1:64	2/0	2/0	2/0	0/2	2/0	2/0
G	1:128	2/0	2/0	2/0	2/0	2/0	2/0
H	1:256	2/0	2/0	2/0	2/0	2/0	2/0
	Titer	<1:2	1:16	1:2	1:64	<1:2	1:8

EXAMPLE 3.

A herpes virus neutralization assay involving 192 sera, was performed following the procedure of Example 1. The total time involved was two man-days. The test required 48 auto-CPE plates and about 500 ml of reagents. To perform the same test by the standard plaque assay (PFU) would have required 20 man-days of work and would have required 2,000 CPE plates and about 2,500 ml of reagents.

WHAT WE CLAIM IS:—

1. A method of determining the presence or absence of the cytopathic effect, without optical magnification, in a cell culture that has been infected with a virus, comprising incubating the infected cell culture for a time sufficient to permit of development of cytopathic effect, staining the cell culture with a protein stain effective to stain and fix the cells substantially immediately, exposing the stained cell culture to a light source and determining whether the cell culture contains a clear area through which light passes.

2. A method according to Claim 1, in which the protein stain is carbolfuchsin stain.

3. A method of carrying out a viral assay, without optical magnification, in a plurality of cell cultures that have been infected with differing predetermined levels of a virus, comprising incubating the infected cell cultures until there is no substantial change in the number of cell cultures showing a cytopathic effect, and staining the cultures with a protein stain effective to stain and fix the cell cultures substantially immediately, whereby the stained fixed cultures can be examined without optical magnification to see whether they contain a clear area through which light passes.

4. A viral assay method comprising

(a) automatic dilution of virus samples;
(b) automatic delivery of the virus sample to a cell suspension;
(c) concurrent infection and formation of cell sheet during incubation of the cells in the suspension;
(d) draining fluids from the culture; and
(e) staining with a protein stain effective to stain and fix the cells substantially immediately and determination of the cytopathic effect without optical magnification.

5. A method according to any one of Claims 1 to 4, in which the virus is rubella.

6. A method according to any one of Claims 1 to 4, in which the virus is mumps.

7. A method according to any one of Claims 1 to 4, in which the virus is measles.

8. A method according to any one of Claims 1 t 4, in which the virus is herpes.

9. A method of determining antibody content of serum comprising the steps of

(a) automatic dilution of the serum samples;

(b) automatic addition of a challenge virus to the samples;

(c) preincubation of the serum/virus mixtures;

(d) automatic delivery of the serum/virus mixtures to a cell suspension;

(e) concurrent infection and formation of cell sheet during incubation of the cells in the suspension;

(f) draining fluids from the culture; and

(g) staining with a protein stain effective to stain and fix the cells substantially immediately and determination of the cytopathic effect without optical magnification.

10. A method according to Claim 1 substantially as hereinbefore described in Examples 1 or 3.

11. A method according to Claim 4 substantially as hereinbefore described in Example 2.

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